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Epstein–Barr Virus Coopts Lipid Rafts to Block the Signaling and Antigen Transport Functions of the BCR

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Summary

The B cell antigen receptor (BCR) functions to initiate signaling and to internalize antigen for processing from within Lyn kinase-enriched membrane lipid rafts. The signaling function of the BCR is blocked by Epstein–Barr Virus (EBV) latent membrane protein 2A (LMP2A), which is constitutively phosphorylated by Lyn. Here, we show that LMP2A resides in lipid rafts and excludes the BCR from entering rafts by Lyn-dependent mechanisms, thus blocking both BCR signaling and antigen transport. Mutant LMP2A that permits BCR signaling and raft translocation still blocks antigen trafficking, indicating independent control of these BCR functions. Thus, EBV coopts the lipid rafts to disarm both the signaling and antigen-processing functions of the BCR by independent mechanisms.

Introduction

The B cell antigen receptor (BCR) plays two critical roles in the B cell response to T cell–dependent antigens: initiating a signal transduction cascade leading to the transcriptional activation of a variety of genes associated with B cell activation and targeting antigen to endocytic compartments where the antigen is processed and presented with MHC class II molecules recognized by helper T cells (reviewed by Parker, 1993; Watts, 1997). The BCR is composed of a surface Ig (sIg) noncovalently associated with the heterodimer Igα/β that functions as the signaling component of the complex (reviewed by Reth and Wienands, 1997). Antigen is recognized by sIg, and cross-linking of the BCR by multivalent antigen leads to the phosphorylation of immune receptor tyrosine-based activation motifs (ITAMs), present on the cytoplasmic domains of the Igα/β chains, by Lyn and other Src family kinases. The phosphorylated ITAMs recruit the SH2 domain–containing kinase Syk, which in turn recruits a variety of signaling molecules, leading to the initiation of signaling cascades resulting in Ca^{2+} mobilization, transcription of gene products associated with B cell activation, and cell proliferation (Cambier et al., 1994; Gold and DeFranco, 1994; Pleiman et al., 1994; Reth and Wienands, 1997).

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Signaling through the BCR alone is not sufficient to mount a B cell response to T cell–dependent antigens (reviewed by Parker, 1993). In addition, B cell activation requires interactions of the B cell with $CD4^+$ helper T cells, initiated by the T cell antigen receptor (TCR) engagement of antigenic peptide–MHC class II complexes on the surface of B cells (Germain, 1994). The BCR plays a key role in the processing and presentation of antigen to helper T cells by efficiently internalizing antigen into intracellular compartments where peptide–MHC class II complexes are assembled (reviewed in Watts, 1997). Current evidence indicates that the signaling and targeting functions of the BCR are interrelated and that signaling through the BCR influences its internalization (reviewed in Wagle et al., 2000). Cross-linking of the BCR by multivalent antigen or anti-Ig accelerates BCR internalization and increases the level of degradation of the BCR compared with BCR-recognizing monovalent antigen (Song et al., 1995; Cheng et al., 1999b). The accelerated transport can be blocked by suppressing BCR signaling using inhibitors of kinases that play a role in B cell activation (Wagle et al., 1998). Signaling domains on Igα/β have been shown to strongly influence the rate of antigen targeting (Aluvihare et al., 1997). In addition, BCR that lack both Igα and Igβ are not correctly targeted to antigen-processing compartments (Shaw et al., 1990; Mitchell et al., 1991; Grupp et al., 1993; Mitchell et al., 1995). The molecular mechanisms by which BCR signaling and antigen targeting are linked are not known at present.

A mechanism by which signaling and antigen-targeting functions of the BCR might be coordinated was recently provided by the description of cholesterol- and sphingolipid-rich plasma membrane microdomains termed lipid rafts. Rafts are proposed to function as platforms for both receptor signaling and trafficking (Brown and Rose, 1992; Simons and Ikonen, 1997; Kurzchalia and Parton, 1999), and we recently provided evidence for lipid rafts in BCR signaling and antigen targeting (Cheng et al., 1999a). Following cross-linking, the BCR was shown to rapidly translocate into Lyn-enriched, CD45-deficient lipid rafts, where both the BCR and Lyn become phosphorylated. Subsequently, the BCR targets bound antigen to the endocytic MHC class II peptide-loading compartments. Targeting appears to be initiated from the lipid rafts, as a portion of the raft constituent sphingolipid G_{M1} is internalized along with the BCR and trafficked to the MHC class II compartments. Thus, current evidence indicates that lipid rafts function as platforms for both the signaling and antigen-targeting functions of the BCR.

Given the importance of the signaling and antigen-trafficking functions of the BCR in B cell activation, it is perhaps not surprising that these functions are targets of pathogens that disarm the immune response, allowing persistence of the organism. EBV establishes a persistent latent infection in a population of human resting B cells that recent evidence indicates resembles memory B cells (Miyashita et al., 1995; Miyashita et al., 1997; Babcock et al., 1998). During latent infection, the viral

gene product LMP2A is expressed on the B cell surface (reviewed in Longnecker and Miller, 1996). Indeed, LMP2A is the only viral protein that is consistently expressed in latently infected resting B cells (Qu and Rowe, 1992; Tierney et al., 1994; Chen et al., 1995; Miyashita et al., 1997). LMP2A is a 12 transmembrane–spanning integral membrane protein with cytoplasmic amino- and carboxy-terminal domains (Laux et al., 1988, 1989; Sample et al., 1989). The 119 amino acid amino-terminal domain of LMP2A contains a variety of motifs involved in protein–protein interactions, including eight tyrosine residues, two of which form an ITAM (Miller et al., 1994b; Longnecker, 2000). LMP2A binds Lyn and the ZAP-70 family tyrosine kinase Syk, and the phosphorylation of LMP2A correlates with its association with Lyn and Syk (Longnecker and Miller, 1996). Tyrosine residue Y112 and residues Y74 and Y85, which form the ITAM, have been identified as being necessary for the binding of LMP2A to Lyn and Syk, respectively (Fruehling and Longnecker, 1997; Fruehling et al., 1998). The association of LMP2A with Lyn and Syk has been proposed to prevent these essential tyrosine kinases from participating in BCR signal transduction (Miller et al., 1995). Indeed, cross-linking of the BCR in LMP2A-containing cells does not lead to phosphorylation of signaling molecules or Ca^{2+} mobilization. BCR signaling is restored by mutations of tyrosine Y112 within the cytoplasmic domain of LMP2A that preclude LMP2A binding to Lyn (Miller et al., 1993; Miller et al., 1994b; Fruehling et al., 1998). Signaling through LMP2A in the absence of a functioning BCR has been shown in a transgenic model to substitute for at least a subset of the signals that allow B cell development and survival (Caldwell et al., 1998, 2000). However, at present, the nature of the LMP2A signal and its relationship to BCR signaling remain to be defined.

Here, we show that LMP2A is constitutively present in lipid rafts of EBV-immortalized human B cell lines. LMP2A functions in the rafts to block the translocation of the BCR into rafts and to block the subsequent signaling and accelerated internalization of the BCR upon BCR cross-linking. The Y112 mutant LMP2A that cannot bind Lyn allows BCR entry into rafts and signaling from the rafts but blocks accelerated trafficking of the BCR upon cross-linking. These results indicate that the signaling and targeting functions of the BCR rely on distinct molecular mechanisms and that LMP2A has adopted separate strategies to block these two functions of the BCR.

Results

Both Wild-Type and Y112-LMP2A Are Constitutively Present in Rafts

The location on the plasma membrane of LMP2A in untreated B cells and in B cells treated to cross-link the BCR was determined. LMP2A normally associates with the Src family kinase Lyn that is concentrated in lipid rafts, and both the wild-type LMP2A (wt-LMP2A) and a mutant version of LMP2A (Y112-LMP2A), which cannot bind Lyn and does not block BCR signaling, were analyzed (Miller et al., 1994b, 1995; Longnecker and Miller, 1996; Fruehling et al., 1998).

$LMP2A^-$ cells (ES1) or cells expressing wt-LMP2A (721.114) or Y112-LMP2A (Y112) were either untreated or treated to cross-link the BCR, and lipid rafts were isolated as previously described (Cheng et al., 1999a). Equal numbers of cells were incubated on ice with $F(ab')_2$ rabbit anti-human Ig, washed, and incubated with $F(ab')_2$ anti-rabbit Ig to cross-link the BCR. Cells were lysed in 1% Triton X-100 and subjected to discontinuous sucrose density gradient centrifugation, and fractions 4 and 11, previously shown to contain raft and soluble regions of the gradient, respectively (Cheng et al., 1999a), were subjected to SDS-PAGE and immunoblotting, probing for LMP2A. wt-LMP2A, which migrates as a 54 kDa protein (Miller et al., 1995), was constitutively present in the raft regions from wt-$LMP2A^+$ cells and absent in $LMP2A^-$ cells (Figure 1A). Significantly, Y112-LMP2A was also constitutively present in the raft fraction, indicating that association with Lyn is not necessary for LMP2A raft localization. Cross-linking the BCR did not alter the presence of either the wt- or Y112-LMP2A in the rafts (Figure 1A).

The lipid rafts from all three cell types also contained Lyn but excluded the phosphatase CD45, and the location of these proteins was not altered by anti-Ig treatment (Figure 1A). The amount of Lyn recovered on the density gradient from wt-$LMP2A^+$ cells was less than that from either $LMP2A^-$ or Y112-$LMP2A^+$ cells. This appears to be the case even though the amount of protein recovered from the wt-$LMP2A^+$ cells was greater than that from either $LMP2A^-$ or Y112-$LMP2A^+$ cells, as judged by the amount of tubulin present in the soluble fraction (Figure 1A). This observation is consistent with previous results indicating that the level of Lyn is downregulated in wt-LMP2A-containing cells due to a decrease in the relative half-life of Lyn (Ikeda et al., 2000).

Lipid rafts are cholesterol-rich microdomains, and, as such, the integrity of lipid rafts is dependent on cholesterol. To demonstrate that the presence of LMP2A in the gradient fractions that contain lipid rafts was dependent on cholesterol in the membrane, wt-$LMP2A^+$ (721.114) cells were treated with the cholesterol-depleting agent methyl-β-cyclodextrin (MCD) at 37°C and washed to remove MCD–cholesterol complexes. A portion of the cells was incubated in cholesterol-containing media for 4 hr at 37°C to allow cholesterol recovery. The cells were then lysed in Triton X-100, and rafts were isolated by sucrose density gradient centrifugation. The lipid raft fraction was analyzed by SDS-PAGE and immunoblotting, probing for LMP2A and Lyn. Treatment with MCD resulted in a complete loss of LMP2A and Lyn from lipid rafts (Figure 1B). Following cholesterol recovery, both LMP2A and Lyn were detected in the lipid raft fractions in the cells, indicating that the localization of LMP2A within the lipid raft fraction is dependent on membrane cholesterol.

The absolute amount of Y112-LMP2A present in the rafts appears less than that of wt-LMP2A. However, the percent of the total LMP2A present in the rafts of the cells expressing wt-LMP2A and Y112-LMP2A appeared similar. To verify that the presence of LMP2A in the rafts is not dependent on association with Lyn, the amount of LMP2A in the rafts was determined for several additional cell lines expressing either the wt-LMP2A or Y112-LMP2A (Figure 2). Quantification of the amount of LMP2A in the raft and soluble regions of the sucrose gradients showed that the percent of LMP2A in the rafts

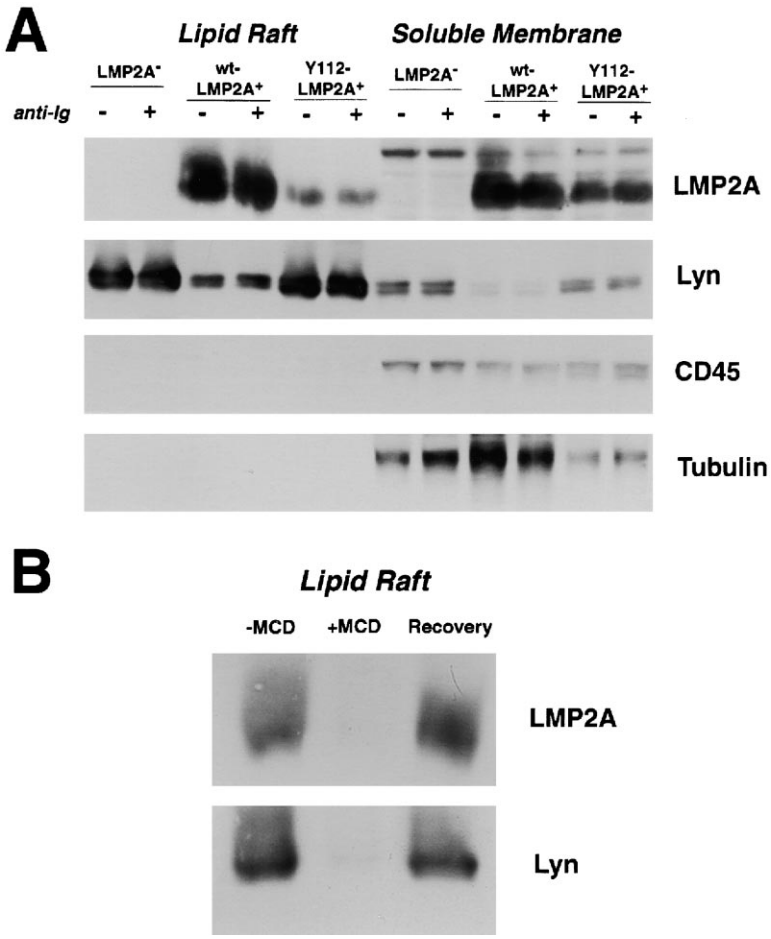


Figure 1. Both WT- and Y112-LMP2A Constitutively Localize to Lipid Rafts

(A) Equivalent numbers of LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells were lysed at 4°C in 1% Triton X-100 in TNE with phosphatase inhibitors, and the lysates were subjected to discontinuous sucrose gradient centrifugation. Fractions 4 and 11, previously determined to correspond to the membrane raft fraction and soluble fraction (Cheng et al., 1999a), were subjected to 10% SDS-PAGE and immunoblotting, probing with antibodies specific for LMP2A, Lyn, and CD45, followed by secondary antibodies and ECL. The soluble fraction contains the majority of cellular proteins, and therefore, to equalize protein concentration, one fourth of the volume of fraction 11 was loaded, as compared with that of fraction 4. Representative blots from at least three separate experiments are shown.

(B) WT-LMP2A$^+$ cells were untreated or treated with MCD (12.5 mM) for 16 min at 37°C and washed to remove drug–cholesterol complexes as previously described (Cheng et al., 1999a). A portion of the cells was removed and placed in cholesterol-containing media for 4 hr to allow for cholesterol recovery. Raft and soluble membrane fractions were obtained and analyzed, as in (A), probing for LMP2A and Lyn. The blots shown are representative of at least four experiments.

varied little, averaging 18% of the total LMP2A for all cell lines (Figure 2). In contrast, less than 1% of CD45 is present in the same raft fractions, indicating that the presence of LMP2A in the rafts was selective and not due to nonspecific contamination of the rafts by membrane proteins. Similarly, tubulin was not detected in the raft fractions of the gradient (data not shown). Thus, mutation of the Lyn binding site in the LMP2A cytoplasmic domain did not appear to significantly alter its raft localization.

In all cell lines analyzed, a significant portion of both wt-LMP2A and Y112-LMP2A was present in the soluble region of the gradient (Figures 1 and 2). It is unclear whether the presence of LMP2A in the soluble region of the gradient reflects the inefficiency of raft isolation in these cells under the conditions used or if LMP2A is actually present both in and out of lipid rafts on the cell surface. However, the distribution of LMP2A in the gradient is similar to that reported for influenza virus HA, which is considered to be localized exclusively in lipid rafts (Scheiffele et al., 1997), suggesting that the raft isolation is somewhat inefficient.

The Effect of LMP2A on BCR Translocation into Rafts

To determine the effect of wt-LMP2A and Y112-LMP2A on the translocation of the BCR into lipid rafts, cells of the LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114) and Y112-LMP2A$^+$ (Y112) lines were incubated with [^{125}I]Fab anti-Ig to label the BCR in the presence or absence of cross-linking antibodies. The cells were lysed and raft and soluble fractions isolated. The position of the BCR in the gradient fractions was quantified by measuring the amount of [^{125}I]Fab anti-Ig. The amount of BCR in lipid rafts from LMP2A$^-$ cells increased ~3-fold upon cross-linking (Figure 3A), consistent with results previously obtained with mouse B cell lines (Cheng et al., 1999a). The presence of wt-LMP2A in lipid rafts blocked the translocation of the BCR into rafts. However, the BCR efficiently translocated into lipid rafts containing Y112-LMP2A. To establish the generality of the finding, the translocation of the BCR into lipid rafts was analyzed for all eight of the cell lines described in Figures 1 and 2. Horseradish peroxidase (HRP)-anti-Ig was used to cross-link the BCR, and the HRP activity in the gradient fractions was quantified (Figure 3B). HRP activity was localized in the raft regions (fractions 3–6) of the LMP2A$^-$ (ES1) and each of the Y112-LMP2A$^+$ cell lines (F.10, C.3, and Y112). In contrast, the BCR in each of the wt-LMP2A$^+$ cell lines (721.114, LCL1, WT.1, and WT.3) was found in the soluble regions of the gradient (fractions 9–12). The BCR in the raft fractions following cross-linking was dependent on the affinity of the BCR for lipid rafts and not on an artifact of aggregation, as CD45,

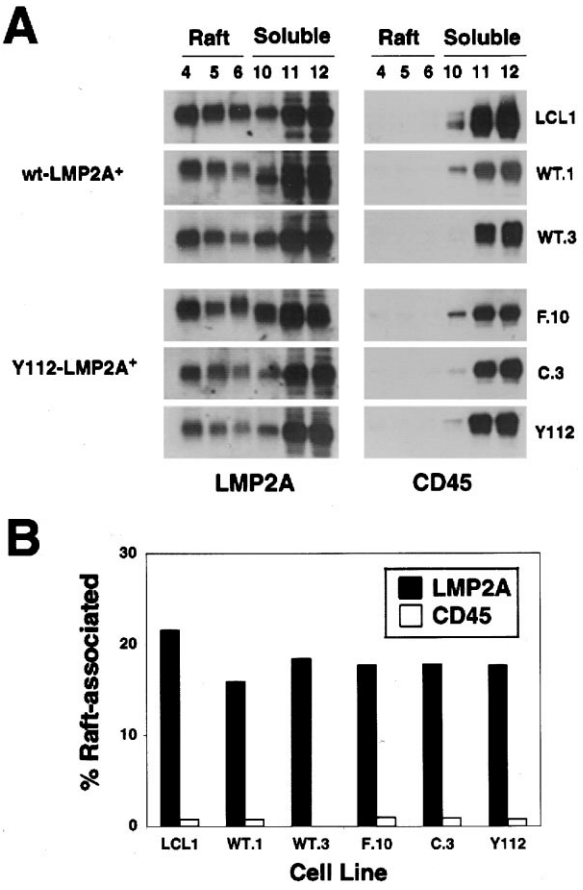


Figure 2. The Ability of LMP2A to Bind Lyn Does Not Influence Raft Localization

(A) Equivalent numbers of cells of the wt-LMP2A-expressing cell lines LCL1, WT.1, and WT.3 or the Y112-LMP2A-expressing cell lines F.10, C.3, and Y112 were lysed and subjected to discontinuous sucrose gradient centrifugation as in Figure 1. Fractions 4–6, containing the insoluble raft membranes, and fractions 10–12, containing the soluble membranes, were subjected to SDS-PAGE and immunoblotting, probing for LMP2A or CD45. To equalize protein concentrations, one fifth of the volume of fractions 10–12 was loaded, as compared with fractions 4–6.

(B) The amount of LMP2A and CD45 in each fraction was quantified by densitometry, and the results are expressed as a percent of the total LMP2A or CD45 present in the raft regions. Shown is a representative immunoblot of two independent experiments and the quantification of that blot.

which is excluded from lipid rafts, remains exclusively in the soluble fraction following cross-linking using CD45-specific antibodies (Figure 3C).

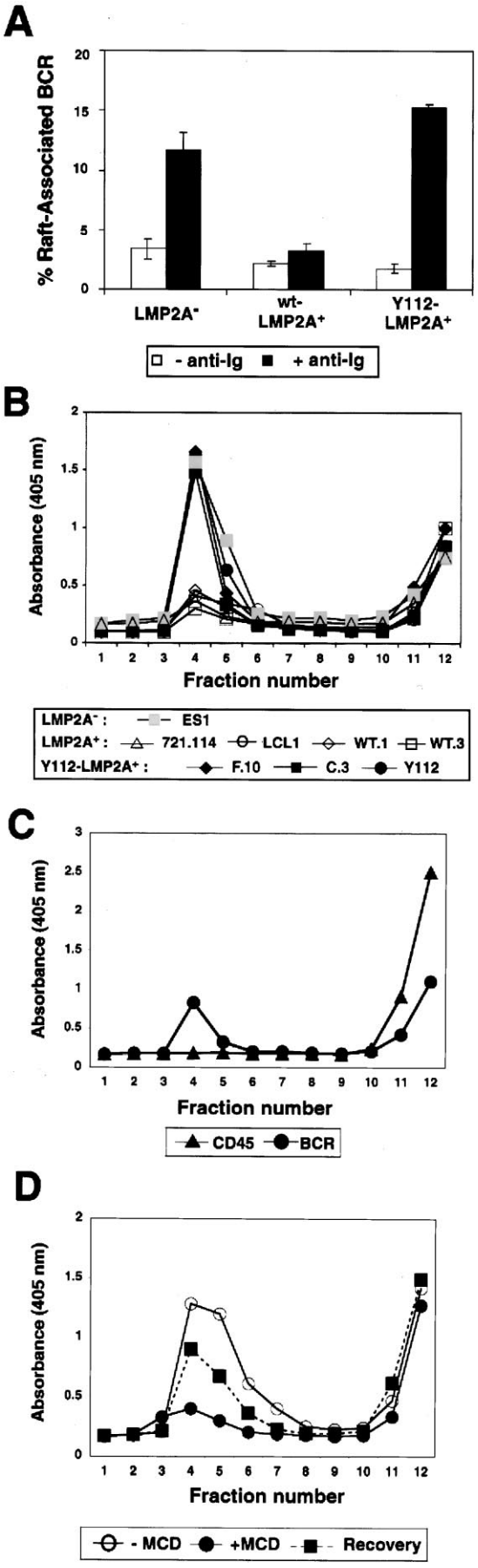
To confirm that the presence of the BCR in Triton X-100–insoluble fractions of the gradient was due to the association of the BCR with rafts, cells were treated with MCD to disrupt lipid rafts before lysis and raft isolation. LMP2A$^-$ (ES1) cells were incubated with MCD, and a portion of the MCD-treated cells was washed and incubated for 4 hr at 37°C in cholesterol-containing media to allow for cholesterol recovery. The cells were treated to cross-link the BCR using HRP-anti-Ig, lysed, and subjected to discontinuous sucrose density centrifugation. The HRP activity was measured in each of the gradient fractions (Figure 3D). HRP activity was present in the raft regions of the gradient obtained from cells not treated with MCD. Treatment with MCD resulted in the loss of HRP activity from the raft regions of the gradient, indicating that the integrity of lipid rafts is necessary for BCR localization to the Triton X-100–insoluble gradient fractions. The effect of MCD was reversible, in that HRP activity was detected in raft regions of gradients obtained from cells allowed to recover from MCD treatment by incubation for 4 hr in cholesterol-containing media.

The Effect of WT-LMP2A and Y112-LMP2A on the Accumulation of Raft-Associated Phosphorylated Proteins following BCR Cross-Linking

Previous studies showed that BCR translocation into rafts resulted in the tyrosine phosphorylation of raft proteins, including Igα and Lyn (Cheng et al., 1999a). To explore the effect of LMP2A on the tyrosine phosphorylation of raft-associated proteins, LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells were treated to cross-link the BCR, lysed, and subjected to sucrose density gradient centrifugation. Raft fractions were analyzed by SDS-PAGE and immunoblotting, probing with a phosphotyrosine-specific mAb (Figure 4A). In the absence of BCR cross-linking, the LMP2A$^-$ and Y112-LMP2A$^+$ cells showed an overall higher constitutive level of tyrosine-phosphorylated proteins in the raft fraction, as compared with wt-LMP2A$^+$ cells. The decrease in steady-state phosphorylation in the raft fraction of wt-LMP2A$^+$ cells is likely due to the reduction in Syk and Lyn kinase activity as previously described (Miller et al., 1994a, 1995). Cross-linking the BCR resulted in an increase in both the number of phosphorylated proteins as well as in the overall level of protein phosphorylation in the rafts from LMP2A$^-$ cells. The low levels of tyrosine phosphorylation in the rafts from wt-LMP2A$^+$ cells were unchanged by BCR cross-linking. However, LMP2A$^+$ cells do contain the machinery capable of tyrosine phosphorylation, as pervanadate treatment that stimulates general protein phosphorylation resulted in a modest but significant increase in the number and level of tyrosine-phosphorylated proteins in the rafts (Figure 4B). The tyrosine phosphorylation pattern in rafts obtained from Y112-LMP2A$^+$ cells resembled that in rafts from LMP2A$^-$ cells, with an increase in the level of phosphorylation in rafts upon cross-linking (Figure 4A). However, the phosphorylation patterns between LMP2A$^-$ and Y112-LMP2A$^+$ cells were not identical, indicating that, although the BCR initiates signaling in both LMP2A$^-$ and Y112-LMP2A$^+$ cells, the resulting cascades may not be identical.

Both WT- and Y112-LMP2A Inhibit the Accelerated Internalization of BCR upon BCR Cross-Linking

To determine the effect of wt- and Y112-LMP2A on BCR internalization and degradation, the internalization of [^{125}I]Fab anti-Ig was followed in LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells. The BCR was labeled with [^{125}I]Fab at 4°C, and the cells were untreated or treated with anti-Ig to cross-link the BCR. The cells were washed and warmed at 37°C for various lengths of time. The radioactivity present inside the cells (internalized) or released from the cells as trichloroacetic acid (TCA)-soluble material (degraded) was measured



and presented as a percentage of the total radioactivity associated with the cells before warming (Figure 5). The BCR in LMP2A$^-$ cells was internalized at an accelerated rate upon anti-Ig treatment, with maximal levels of BCR internalization (35%) achieved at 10 min after warming. In the absence of cross-linking, similar levels of BCR internalization were not achieved until nearly 60 min of incubation at 37°C. Significantly, the presence of wt-LMP2A in cells blocked the accelerated internalization of the BCR upon cross-linking. Similarly, BCR-accelerated internalization was blocked by Y112-LMP2A (Figure 4). In both wt-LMP2A$^-$ and Y112-LMP2A$^+$ cell lines, the maximal levels of BCR internalization ($\sim$20%) were lower than those obtained with LMP2A$^-$ cells.

In addition to accelerating the rate of BCR internalization, BCR cross-linking in LMP2A$^-$ cells resulted in a larger fraction of BCR (15% by 40 min) being degraded, as compared with untreated cells (Figure 5). In cells containing wt-LMP2A, cross-linking the BCR had no effect on BCR degradation. In contrast, in the presence of Y112-LMP2A, the fraction of the BCR degraded upon cross-linking was significantly increased and reached levels similar to that in LMP2A$^-$ cells. Taken together, these results indicate that both the wt- and Y112-LMP2A blocked accelerated internalization, but only the wt-LMP2A blocked degradation, indicating that these two BCR activities were independently regulated.

The Role of Src Family Kinases in BCR Signaling, Raft Translocation, and Internalization

The results described above indicate that the inability of Y112-LMP2A to associate with Lyn allowed BCR translocation into lipid rafts and subsequent degradation of the BCR. This observation suggested a relationship between Lyn kinase activity and these BCR functions. To further delineate the requirements for kinase activity for BCR raft translocation, the effect of the Src

Figure 3. WT-LMP2A Blocks BCR Raft Translocation

(A) The BCR on LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells was labeled with [^{125}I]Fab anti-Ig and cross-linked or not with HRP-anti-Ig at 4°C. Cells were lysed and rafts isolated by discontinuous sucrose density gradient centrifugation. The cpm were measured in each fraction, and the amount present in raft fractions 3–6 was expressed as the percent of the total cell-associated ^{125}I. Plotted are means and SEM from at least three independent experiments.

(B) The location of the BCR following cross-linking in each of the eight cell lines shown in Figures 1 and 2 was determined. The BCR was cross-linked using HRP-anti-Ig at 4°C, the cells lysed, and the rafts and soluble membrane fractions isolated as in (A). The HRP activity in the fractions is given and is representative of at least two separate experiments.

(C) LMP2A$^-$ cells were treated with HRP-anti-Ig or HRP-anti-CD45 prior to raft isolation by sucrose density gradient centrifugation. The HRP activity in each fraction was measured. Shown is a representative of three independent experiments.

(D) LMP2A$^-$ cells were untreated or pretreated with MCD (12.5 mM) for 16 min at 37°C and washed to remove drug–cholesterol complexes. Half of the cells were allowed to recover by incubation in cholesterol-containing media for 4 hr. The cells were treated with HRP-anti-Ig, lysed, and the lysates subjected to discontinuous sucrose gradient centrifugation. The HRP activity in each fraction was measured. The data shown represent at least three independent experiments.

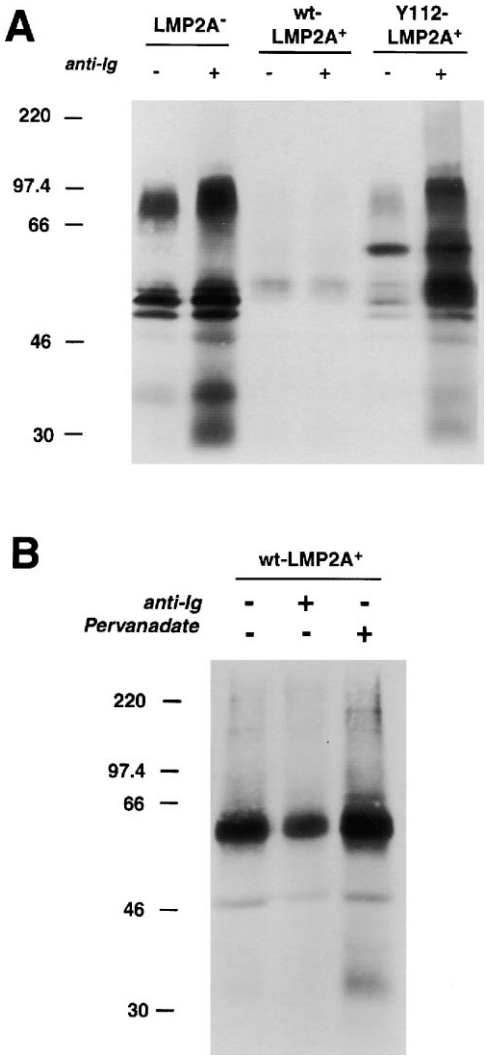


Figure 4. Phosphorylated Proteins Do Not Accumulate in Rafts of WT-LMP2A$^+$ Cells upon BCR Cross-Linking

(A) LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells were untreated or treated with anti-Ig, and raft fractions were isolated and subjected to 10% SDS-PAGE, as described in Figure 1. The resulting gels were transferred to polyvinylidenedifluoride (PVDF) and immunoblotted, probing with an HRP-labeled phosphotyrosine-specific antibody, followed by ECL.

(B) Rafts were isolated by discontinuous sucrose gradient centrifugation from wt-LMP2A$^+$ (721.114) cells untreated or treated with anti-Ig or pervanadate. Gradient fraction 4 was subjected to 10% SDS-PAGE and immunoblotting, probing with an HRP-labeled phosphotyrosine-specific antibody, followed by ECL. Shown are representative blots from at least three independent experiments.

family kinase inhibitor PP2 (Hanke et al., 1996) on BCR translocation was assessed. LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells were preincubated with 50 or 100 μM PP2, and the BCR was labeled with [^{125}I]Fab and cross-linked with anti-Ig in the continued presence of PP2. The cells were lysed and subjected to discontinuous sucrose density centrifugation. The percent of BCR present in lipid rafts was determined by measuring the counts per minute (cpm) associated with raft fractions 3–6.

To ensure that incubation with PP2 inhibited BCR signaling upon cross-linking, raft fractions were subjected to SDS-PAGE and immunoblotting, probing with a phosphotyrosine-specific mAb. BCR cross-linking in untreated cells resulted in an increase in phosphorylation in the rafts of LMP2A$^-$ and Y112-LMP2A$^+$ cells but not in the rafts of wt-LMP2A$^+$ cells (Figure 6A). Treatment with PP2 reduced the levels of raft-associated phosphorylation following BCR cross-linking in LMP2A$^-$ and Y112-LMP2A$^+$ cells.

Upon cross-linking in the absence of PP2, the BCR translocated into lipid raft regions in LMP2A$^-$ and Y112-LMP2A$^+$ but not in wt-LMP2A$^+$ cells. Incubation with PP2 did not inhibit the cross-linking-induced translocation of BCR into lipid raft regions in either LMP2A$^-$ or Y112-LMP2A$^+$ cells, indicating that BCR signaling through Src family kinases is not necessary for BCR raft translocation (Figure 6B). Indeed, the percent of the BCR in lipid rafts was increased in anti-Ig-treated cells in the presence of PP2, suggesting that translocation or stable residency in rafts may be negatively regulated by Src family kinases. Incubation with PP2 had no effect on BCR raft localization in wt-LMP2A$^+$ cells, and only a small percentage of BCR was isolated in the raft regions of these cells under all conditions.

The effect of PP2 on the internalization and degradation of the BCR was analyzed. The accelerated internalization of BCR induced by cross-linking was blocked in LMP2A$^-$ cells by incubation with either 50 or 100 μM PP2 (Figure 6C), reducing the levels of BCR internalization to constitutive levels. PP2 treatment had no effect on the internalization of BCR in wt-LMP2A$^+$ (data not shown) or Y112-LMP2A$^+$ cells (Figure 6C), neither of which internalized their BCR in an accelerated fashion upon BCR cross-linking.

Treatment with PP2 inhibited degradation of the BCR in LMP2A$^-$ cells in a dose-dependent fashion, although degradation was not reduced to constitutive levels at the highest concentration tested (100 μM) (Figure 6C). Treatment of Y112-LMP2A$^+$ cells inhibited BCR degradation upon cross-linking to levels resembling basal degradation (Figure 6C). Incubation with PP2 had no effect on BCR degradation in anti-Ig-treated wt-LMP2A$^+$ cells (data not shown), which did not show increased degradation of BCR upon cross-linking. Taken together, these results indicate that accelerated targeting and degradation of the BCR are dependent on kinase activity but that the signaling requirements for targeting and degradation are not identical.

Discussion

Recently, attention has focused on sphingolipid- and cholesterol-rich plasma membrane microdomains or lipid rafts and their roles in signal transduction and membrane trafficking in immune cells. Current evidence suggests that lipid rafts function as platforms for both signaling and trafficking of the multichain immune recognition receptors, including the BCR, the TCR, and the IgE receptor (reviewed in Langlet et al., 2000). For B cells, the rafts have been shown, thus far, to concentrate the Src family kinase Lyn and a newly discovered Src family kinase regulator, Cbp (Brdicka et al., 2000; Kawa-

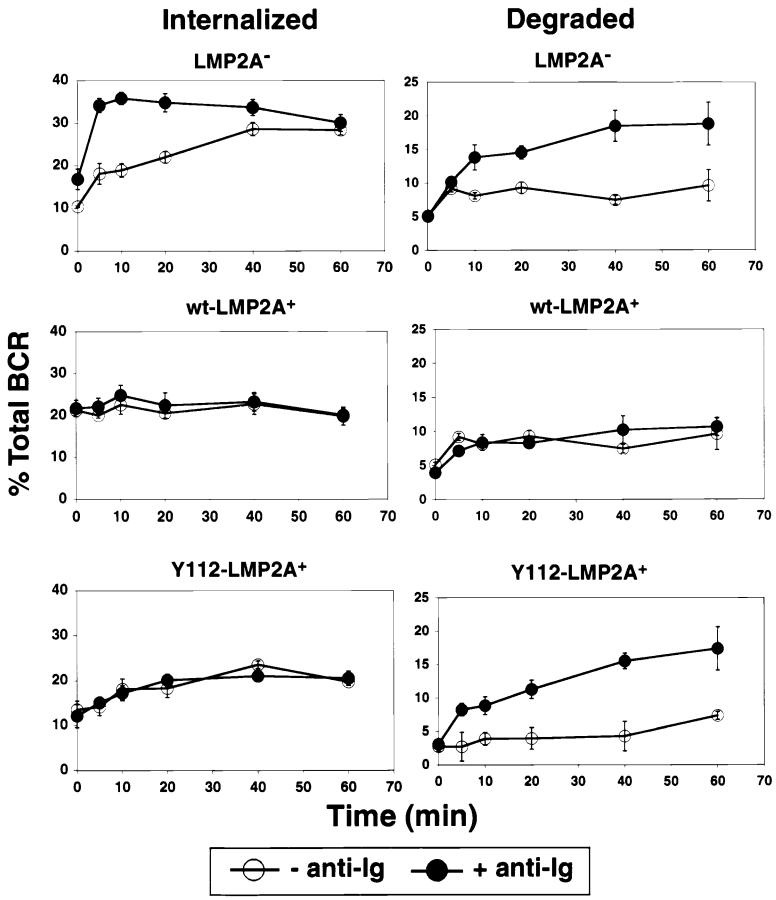


Figure 5. The WT- and Y112-LMP2A Differentially Affect BCR Internalization

The BCR on LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells were labeled with [^{125}I]Fab anti-Ig and cross-linked or not with F(ab′)$_2$ anti-Ig at 4°C. The cells were washed and incubated at 37°C for various lengths of time. At the end of each time point, the cells were washed, saving the supernatant, and acid stripped to remove [^{125}I]Fab remaining on the cell surface. The radioactivity associated with the cells following stripping was measured and taken as the internalized fraction. The incubation media was treated with TCA and centrifuged to remove precipitates. The cpm associated with TCA-soluble material were measured and taken as the degraded fraction. The internalized and degraded fractions are expressed as the percent of the total radioactivity associated with the cells at each time point before washing and stripping. Plotted are means and SEM from at least three independent experiments.

buchi et al., 2000), and to exclude the phosphatase CD45. It is likely that other key elements of the BCR signaling pathway also are concentrated in rafts. As depicted in Figure 7, following antigen-induced cross-linking, the BCR rapidly translocates into lipid rafts, where it is phosphorylated and subsequently internalized from the raft into endocytic compartments in the cell. BCR internalized from the rafts has been shown to be rapidly transported to MHC class II–containing compartments, where antigen peptide–MHC class II complexes are assembled (Song et al., 1995; Cheng et al., 1999a, 1999b). Internalization may also result in receptor degradation, resulting in BCR downregulation and cessation of signaling. Here, we provide evidence that a B cell tropic virus, EBV, targets not the BCR directly but rather the B cell lipid raft to disrupt both the signaling and antigen-targeting functions of the BCR. The coopting of the lipid raft by a pathogenic virus lends additional support for the physiological relevance of the rafts in B cells. In addition, the mechanisms by which EBV blocks BCR function from within rafts have allowed a further dissection of the BCR signaling and trafficking pathways.

The molecular mechanisms underlying BCR raft translocation, signaling, and transport of antigen are, at present, not well defined. The results presented here, investigating the effects of both a wild-type and mutant LMP2A as well as a specific Src kinase inhibitor on BCR translocation into rafts and internalization, provided several insights into the mechanisms underlying BCR functions. A summary of the findings is depicted in Figure 7.

The first observations involve the molecular requirements for BCR raft translocation. Following cross-linking, the BCR was not associated with rafts in cells expressing wt-LMP2A. We interpret this to represent a wt-LMP2A-induced block in BCR translocation and refer to it as such, although it is possible that the BCR translocates into the raft but is only weakly associated with the raft. The physical presence of LMP2A in rafts was not sufficient to block BCR entry into rafts, since BCR translocated into rafts containing Y112-LMP2A. Since Y112-LMP2A does not associate with Lyn or block BCR signaling, it is tempting to suggest that the block in BCR translocation by wt-LMP2A was due to its inhibition of BCR signaling. However, the Src family kinase inhibitor PP2 that blocked BCR signaling did not block BCR translocation into rafts. Conversely, PP2 did not permit raft translocation of the BCR in wt-LMP2A$^+$ cells. A higher percentage of BCR was isolated in lipid rafts in LMP2A$^-$ and wt-LMP2A$^+$ cells after treatment with PP2, suggesting a possible negative regulation of BCR translocation by Src family kinases. Thus, the initiation of BCR signaling through Src family kinases is not required for BCR raft translocation, and the inhibition of raft translocation by wt-LMP2A is not due to the effect of wt-LMP2A on BCR signaling. The results also indicate that Lyn activity is not required for stable residency of either LMP2A or the translocated BCR in the lipid rafts.

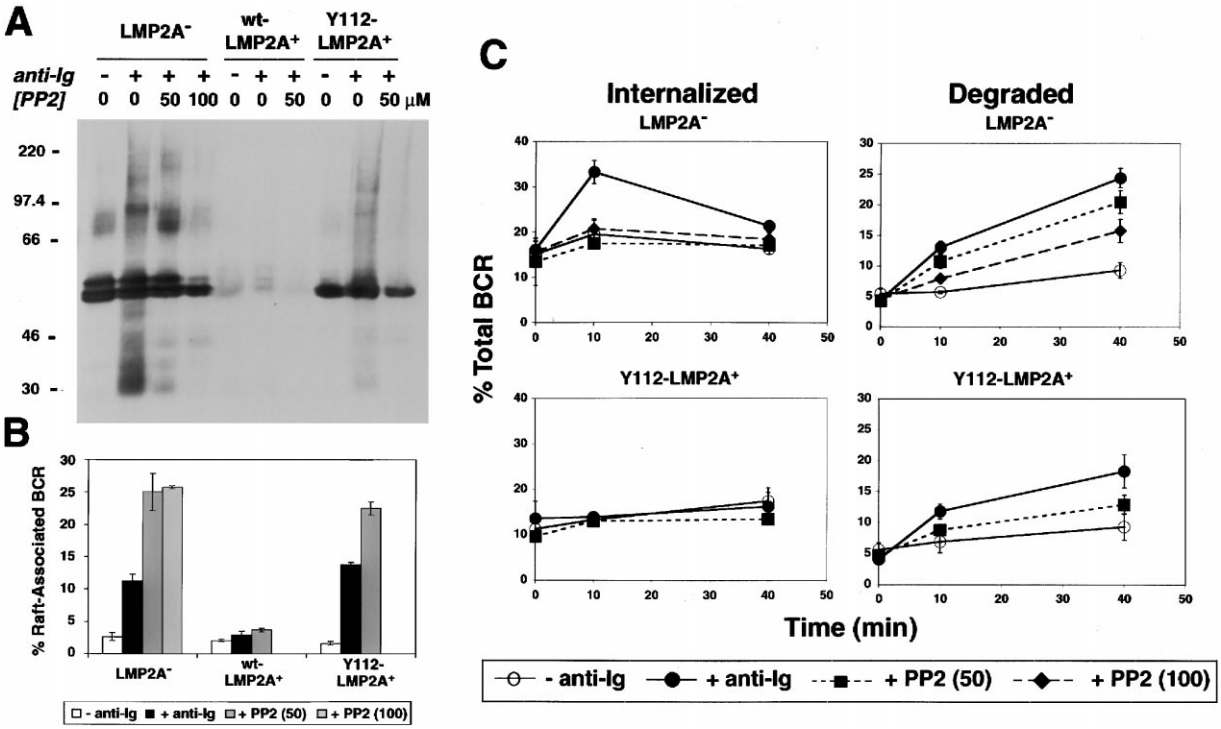


Figure 6. The Effect of the Src Family Kinase Inhibitor PP2 on BCR Raft Translocation and Internalization

(A) The BCR on LMP2A$^-$ (ES1), wt-LMP2A$^+$ (721.114), and Y112-LMP2A$^+$ (Y112) cells were labeled with [^{125}I]Fab anti-Ig. The cells were treated with anti-Ig to cross-link the BCR in the presence or absence of 50–100 μM PP2. Raft fractions were isolated and subjected to 10% SDS-PAGE and immunoblotting, as described in Figure 1, probing with an HRP-labeled phosphotyrosine-specific antibody, followed by ECL. Shown is a representative blot from at least two independent experiments.

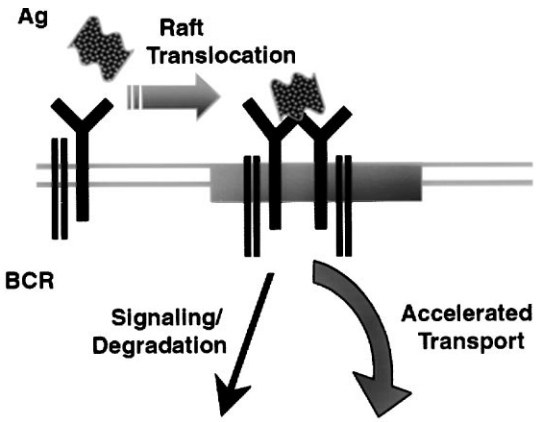
(B) The cpm were measured in the fractions from (A), and the amount present in raft fractions 3–6 was expressed as the percent of the total cell-associated ^{125}I. Plotted are means and SEM from at least two independent experiments for each cell type.

(C) BCR internalization and degradation was followed as described in Figure 5 in LMP2A$^-$ (ES1) and Y112-LMP2A$^+$ (Y112) cells treated with anti-Ig in the presence or absence of 50–100 μM PP2. Plotted are means and SEM from at least three independent experiments.

The results presented here also address the requirements for BCR antigen targeting. The BCR was not internalized at an accelerated rate from the cell surface unless BCR raft translocation and signaling occurred. Thus, in the presence of wt-LMP2A or of the inhibitor PP2, both of which blocked BCR signaling, the BCR was cleared from the surface at a slow constitutive rate. However, BCR raft translocation and signaling were not sufficient for accelerated internalization of the BCR, since Y112-LMP2A, which did not block BCR raft translocation or BCR signaling, blocked BCR-accelerated internalization. Thus, the accelerated transport of the BCR has requirements in addition to raft translocation and signaling. In addition, LMP2A interferes with BCR internalization by a mechanism that is independent of its association with Lyn.

A previously unappreciated aspect of BCR function suggested by the findings of this study is that the accelerated internalization and increased degradation of the BCR upon cross-linking are separable events. This is evidenced by the observations that Y112-LMP2A blocked BCR-accelerated transport but did not affect the increased degradation of the BCR upon cross-linking. Similarly, in the presence of the inhibitor PP2, accelerated internalization of the BCR was completely blocked, while BCR degradation was only partially inhibited. In all cases, the ability of the BCR to signal was necessary for its increased degradation upon cross-linking. Increased degradation of the BCR may reflect a downmodulation of the BCR in response to signaling. This downmodulation may be separate from the specialized antigen trafficking of the BCR that accompanies accelerated internalization and for which little antigen degradation is measured. Thus, downmodulation and measurable receptor degradation in lysosomes and accelerated transport of antigen to the MHC class II peptide-loading compartments may be distinct outcomes of BCR antigen binding.

A final issue raised by the results presented here is the physiological role of the observed block in BCR function by LMP2A. EBV establishes latency in human hosts in a population of resting B cells, recently described as having a phenotype resembling that of the memory B cell pool (Miyashita et al., 1995, 1997; Babcock et al., 1998). In order to achieve latent infection in this memory-like pool, EBV must either infect memory B cells themselves or, alternatively, drive infected B cells to differentiate into memory B cells. Thus, EBV must provide signals that promote survival of latently infected memory cells. If EBV does not infect memory B cells but drives infected cells to a memory-like state, the virus must, in addition, ensure that signals are provided to



	Raft Translocation	Signaling	Degradation	Accelerated Transport
LMP2A⁻	+	+	+	+
+PP2	+	-	-	-
wt-LMP2A⁺	-	-	-	-
+PP2	-	-	-	-
Y112-LMP2A⁺	+	+	+	-
+PP2	+	-	-	-

Figure 7. LMP2A Targets Lipid Rafts, Blocking Both the Signaling and Antigen Transport of the BCR

Upon cross-linking by antigen, the BCR rapidly translocates into plasma membrane lipid rafts, where signaling and internalization for degradation and antigen transport are initiated. The chart depicts a summary of the effects of wt-LMP2A, Y112-LMP2A, and the Src family kinase inhibitor PP2 on each BCR function. As shown, EBV blocks all steps in the pathway. The results also lead to the conclusions that the BCR translocation into rafts is independent of signaling and that internalization leading to degradation and to accelerated transport for processing are independent outcomes of antigen binding.

allow differentiation of infected cells. In either scenario, the virus must ensure that infected cells are not eliminated by the immune system.

LMP2A is the only viral product that is consistently detected in EBV latently infected cells (Qu and Rowe, 1992; Tierney et al., 1994; Chen et al., 1995; Miyashita et al., 1997), suggesting that it is responsible for establishing the conditions required for EBV persistence during latent infection. Transgenic models have shown that LMP2A provides B cells with development and survival signals that normally proceed from the BCR (Caldwell et al., 1998, 2000). Thus, LMP2A is presumably capable of promoting survival of latently infected B cells. In addition, it is possible that signals initiated from LMP2A could mimic BCR signals normally necessary to drive differentiation to memory B cells in response to antigen encounter.

In addition to providing signals that allow survival and differentiation of B cells, LMP2A may also function in the establishment and persistence of EBV latency by protecting infected cells from surveillance by the immune system. The memory cells in which EBV persists during latent infection are resting B7-negative cells (Miyashita et al., 1997; Babcock et al., 1999) and thus are inherently ineffective as antigen-presenting cells for helper T cells. By blocking BCR signaling, LMP2A ensures persistence in this quiescent state. Moreover, by blocking trafficking of antigen, LMP2A further guarantees a block in antigen presentation. Blocking B cell activation also could have the important repercussion of regulating the number of infected B cells by inhibiting antigen-driven expansion. Further refinement of the model for LMP2A function may add to an understanding of the mechanisms by which EBV persists in human B cells during latent infection.

Experimental Procedures

Cell Lines, Antibodies, and Reagents

The EBV-infected LMP2A$^+$ human lymphoblastoid cell line (LCL) 721.114 (DeMars et al., 1983) was maintained in DME supplemented as previously described (Jelachich et al., 1984) and containing 15% FCS (15% complete media [15% CM]). The EBV-infected LMP2A$^-$ LCL ES1 (Miller et al., 1994b, 1995) and the LCL Y112 (Fruehling et al., 1998), expressing a mutant LMP2A carrying a Y_{112}/F mutation, were maintained in RPMI supplemented as above and containing 15% FCS (15% RPMI). Additional cell lines tested for the raft localization of LMP2A and the BCR were LMP2A$^+$ LCLs LCL1, WT.1, and WT.3 and the Y112-LMP2A$^+$ LCLs C.3 and F.10 (Longnecker et al., 1993; Fruehling et al., 1998). These additional lines were maintained in 15% RPMI.

Fab goat antibodies (specific for human γ chain [Fab anti-Ig]),

F(ab′)$_2$ rabbit antibodies (specific for human IgA, G, and M), HRP-conjugated F(ab′)$_2$ goat antibodies (specific for rabbit IgG), HRP-conjugated goat antibodies (specific for rabbit γ chain), and HRP-conjugated goat antibodies (specific for mouse γ chain) were purchased from Jackson ImmunoResearch (West Grove, PA). The rat mAb specific for LMP2A (14B7) was described previously (Fruehling et al., 1996). Rabbit polyclonal antibodies specific for Lyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The CD45-specific mAb and the HRP-conjugated phosphotyrosine-specific recombinant mAb RC20H were purchased from Transduction Laboratories (San Diego, CA). The rat monoclonal antibody YL1/2, specific for tubulin, was a kind gift from Dr. Douglas T. Fearon (University of Cambridge, UK).

Fab anti-Ig was iodinated using the iodide monochloride method as previously described (Goldstein et al., 1983). Specific activity of the [^{125}I]Fab anti-Ig was 0.5–1.0 $\times$ 10^7 cpm/μg, and the cell binding efficiency was greater than 80%, as demonstrated by competition with unlabeled Fab anti-Ig binding to the surface of LCLs. More than 90% of the [^{125}I]Fab anti-Ig was precipitated by 10% TCA, indicating little free ^{125}I in the preparation.

The Src family kinase inhibitor PP2 was purchased from CalBiochem (San Diego, CA), and a 15 mg/mL stock solution was prepared in DMSO. Pervanadate solution (75 μM Na_3VO_4 and 0.075% H_2O_2) was made fresh for each experiment.

Isolation of Lipid Rafts

Lipid rafts were isolated from LCLs by lysis of cells in Triton X-100 and floatation on sucrose density gradients as previously described (Cheng et al., 1999a). In brief, LCLs (10^8) were incubated for 30 min on ice in 1% Triton X-100 in TNE (10 mM Tris/HCl [pH 7.5], 150 mM NaCl, and 5 mM EDTA) with protease inhibitors (2.5 mg/mL each of chymostatin, leupeptin, antipain, and pepstatin A in DMSO) and phosphatase inhibitors (2 mM Na_3VO_4 and 10 mM NaF). Lysates were homogenized with ten strokes in a Wheaton loose-fitting dounce homogenizer and centrifuged at 900 $\times$ g for 10 min to remove nuclei and cellular debris. Cleared supernatants were diluted 1:1 with 1 ml 85% sucrose in TNE with phosphatase inhibitors and layered at the bottom of a Beckman 14 $\times$ 89 mm centrifuge tube. The lysate was overlayed with 6 ml 35% sucrose in TNE with phosphatase inhibitors and 3.5 ml 5% sucrose in TNE with phosphatase inhibitors. Gradients were centrifuged at 200,000 $\times$ g in an SW41 rotor for 16–20 hr at 4°C, and 1 ml fractions were collected from the top of the gradient.

Internalization of [^{125}I]Fab Anti-Ig

Internalization of surface BCR was measured as previously described (Song et al., 1995). In brief, BCRs were labeled at 4°C in DME containing 6 mg/mL BSA (DME-BSA) with [^{125}I]Fab anti-Ig and cross-linked or not at 4°C with F(ab′)$_2$ rabbit anti-human Ig followed by HRP-conjugated F(ab′)$_2$ goat anti-rabbit Ig. Cells were washed and incubated at 37°C in 15% CM for 0–60 min. At the end of each incubation period, cells were harvested into ice-cold 15% CM and pelleted. Supernatants were collected and treated at 4°C with 10% TCA, and the amount of TCA-soluble radioactivity was measured and taken as the amount of degraded [^{125}I]Fab anti-Ig. Pelleted cells were resuspended in a low-pH solution (20 mM HCl and 150 mM NaCl) and incubated at 4°C for 15 min to strip [^{125}I]Fab anti-Ig from the cell surface. Cells were pelleted, and the radioactivity recovered in the acid wash was taken as the surface fraction. The cell pellet was resuspended in DME-BSA, and the radioactivity associated with the cells after acid stripping was measured and taken as the amount of internalized [^{125}I]Fab anti-Ig.

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